Expression of \textit{nip}_{\textit{P.w}} of \textit{Pectobacterium wasabiae} is dependent on functional \textit{flgKL} flagellar genes

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While flagellum-driven motility is hypothesized to play a role in the virulence of \textit{Pectobacterium} species, there is no direct evidence that genes involved in flagellum assembly regulate the synthesis of virulence factors. The purpose of this study was to identify genes that affect the production or secretion of necrosis-inducing protein (Nip) in the strain SCC3193. Transposon mutagenesis of an RpoS strain overexpressing \textit{nip}_{\textit{P.w}} was performed, and a mutant associated with decreased necrosis of tobacco leaves was detected. The mutant contained a transposon in the regulatory region upstream of the flagellar genes \textit{flgK} and \textit{flgL}. Additional mutants were generated related to the flagellar genes \textit{flaC} and \textit{flaA}. The mutation in \textit{flgK}, but not those in \textit{flaC} and \textit{flaA}, inhibited \textit{nip}_{\textit{P.w}} transcription. Moreover, the regulatory effect of the \textit{flgKL} mutation on \textit{nip}_{\textit{P.w}} transcription was partially dependent on the Rcs phosphorelay. Secretion of \textit{Nip}_{\textit{P.w}} was also dependent on a type II secretion mechanism. Overall, the results of this study indicate that the \textit{flgKL} mutation is responsible for reduced motility and lower levels of \textit{nip}_{\textit{P.w}} expression.

INTRODUCTION

Several \textit{Pectobacterium} species cause soft rot and blackleg in potatoes (Pérombelon, 2002). Soft-rot symptoms are mainly caused by the production of plant cell wall-degrading enzymes (PCWDE), which act as potent virulence factors (Toth & Birch, 2005). This study deals with a well-characterized \textit{Pectobacterium} strain SCC3193, that was recently reclassified as \textit{Pectobacterium wasabiae} (\textit{P.w}). In \textit{P.w}, motility and PCWDE production traits are coupled to, and indispensable for, successful infection of the host plant (Cui et al., 2008; Andresen et al., 2010). Expression of virulence in \textit{P.w} is modulated at transcriptional (Andresen et al., 2007; Cui et al., 2001; Hyytiäinen et al., 2001, 2003; Mukherjee et al., 2000) and post-transcriptional (Chatterjee et al., 1995, 2009; Cui et al., 2008; Köiv et al., 2013) levels, but is also regulated by quorum sensing (for a review, see Pöllumaa et al., 2012). PCWDE have to be secreted to reach their target substrates in the plant cell wall. For that, a type II secretion system, also known as the Out-system, is used in case of pectinases and the cellulase (Reeves et al., 1994), whereas the type I Prt machinery is applied for the secretion of proteases (Marits et al., 1999).

In addition to PCWDE, many plant-pathogenic bacteria exploit certain proteins as toxins to suppress plant defence responses (Espinoso & Alfano, 2004; Friesen et al., 2008). In \textit{P.w}, a toxin-like necrosis-inducing protein (\textit{Nip}_{\textit{P.w}}) was isolated and shown to be essential for different stages of plant-bacterial interactions (Mattinen et al., 2004; Pemberton et al., 2005). When the \textit{nip}_{\textit{P.w}} gene was inactivated in \textit{P.w}, symptoms of soft-rot disease in potato tubers were significantly reduced (Mattinen et al., 2004). Importantly, the deduced sequence of \textit{Nip}_{\textit{P.w}} showed significant homology to necrosis- and ethylene-inducing (Nep1) protein of fungi and oomycetes (Mattinen et al., 2004). The effect of \textit{nip} mutation on virulence was also obvious in case of \textit{Pectobacterium atrosepticum} (\textit{P. atrosepticum}), where the \textit{nip} mutation reduced both potato soft rot and stem rotting (Pemberton et al., 2005). In accordance with suggested toxin-like function of Nep1-like proteins (NLPs), including the \textit{Nip}_{\textit{P.w}}, Ottmann et al. (2009) demonstrated that they accelerate disease progression via disintegration of plant cell plasma membranes. Though the NLPs probably play an important role in pathogenesis, only some data are available on their regulation in bacterial pathogens. In \textit{P. atrosepticum}, the \textit{nip} was proposed to be regulated by the LuxR family regulator EccR, and the global regulator RsmA (Pemberton et al., 2005). In \textit{P.w} strain SCC3193, expression of \textit{nip}_{\textit{P.w}} was reported to be negatively controlled by RpoS (Mattinen et al., 2004).

Alongside the production of certain proteins (e.g. PCWDE and toxic proteins), flagellar motility is an important
virulence determinant in several pathogenic bacteria (Gauer et al., 2007; Matsumoto et al., 2003; Hossain et al., 2005). Intriguingly, in P. w, motility and PCWDE production are coregulated: the master regulator FlhDC of flagellar genes is also implicated in regulation of PCWDE production (Cui et al., 2008; Chatterjee et al., 2009). In addition, we have previously demonstrated that both PCWDE synthesis and motility are regulated by the Rcs phosphorelay (Andersen et al., 2007). The Rcs phosphorelay negatively affects PCWDE expression by inhibiting the expression of flhDC and rsmB (Andersen et al., 2010). In this phosphorelay, the RcsC sensor kinase is autophosphorylated in response to environmental stimuli and activates the response regulator RcsB by phosphoryl group transfer via RcsD, a phosphotransmitter protein.

Within flagella regulons of Salmonella and E. coli, the operons comprise three classes. Class I genes, the flhD and flhC, represent the master regulatory operon responsible for the control over flagellar genes expression (for a review, see Prüft, 2000; Wang et al., 2006). The FlhD and FlhC proteins activate class II genes, which participate in formation of the hook basal body (HBB) structure, synthesis of the sigma factor Flia (\( \sigma^b \)) and the anti-sigma factor FlgM (Liu & Matsumura, 1994). Transcription of class III genes requires the Flia protein, and the products of these genes comprise the flagellar filament and the chemosensory machinery (Karlinsey et al., 2000).

Given that flagellar mutants of human pathogens have pleiotropic phenotypes, it has been hypothesized that genes involved in flagellum assembly also play a role in production and/or secretion of virulence factors, thereby influencing bacterial–host interactions independent of the motility trait (Ghelardi et al., 2002; Konkel et al., 2004; Bouillaut et al., 2005). In the present study, we identify novel mutations that affect expression of the necrosis-inducing protein of P. wasabiae (Nip\(_{P.w}\)) and will show that the flgKL mutation causes both reduced motility and lower levels of nip\(_{P.w}\) expression.

**METHODS**

**Bacterial strains, vectors, transduction, and growth conditions.**

The strains and plasmids used in this study are listed in Table 1. P. w and Escherichia coli strains were grown at 30 °C and 37 °C, respectively. They were grown on LB medium or M9 minimal salts medium containing 10% potato extract (v/v) and appropriate trace elements (Miller, 1972; Sambrook & Russell, 2001). When required, antibiotics were added as follows: ampicillin (Amp) 150 \( \mu \)g ml\(^{-1}\), kanamycin (Km) 100 \( \mu \)g ml\(^{-1}\) and chloramphenicol (Chm) 25 \( \mu \)g ml\(^{-1}\). To induce the hypersensitive response (HR) in tobacco plants, P. w cells were grown for three days at 15 °C on solid minimal induction medium (Huynh et al., 1989) supplemented with 10 mM mannotol and 0.5% sucrose (w/v). Nicotiana tabacum cv. Samsun plants used in the necrosis assays were grown in greenhouses at approximately 22 °C. Plants were used before flowering. Chromosomal markers were transduced with T4GT phage as previously described (Pirhonen et al., 1991).

**DNA manipulations.** Standard DNA techniques described in Sambrook & Russell (2001) were applied. The oligonucleotide primers used for PCR amplifications are listed in Table 2.

**Transposon mutagenesis and construction of mutant strains.**

Transposon mutagenesis of the rpoS strain SCC8003 was performed using \( \phi \) phase 1105 as previously described (Pirhonen et al., 1991). To localize the inserted transposon in motility mutants, arbitrary PCR primers (Caetano-Anolés, 1993) were used in combination with Tn10-specific primers (listed in Table 2) to amplify flanking DNA sequences. Mutated genes were identified and the inserts were localized by sequencing the amplified fragments.

The flIA::Cm, flIC::Cm and outC::Cm fragments were amplified using the primers FlIAp1 and FlIAp2, FlICp1 and FlICp2, OutCP1 and OutCP2 respectively (listed in Table 2), with plasmid pKD3 as template. Chromosomal genes were replaced with mutated genes using the \( \lambda \) Red system (Datsenko & Wanner, 2000).

**Enzyme assays.** For \( \beta \)-glucuronidase (GusA) assays, cells were grown at 28 °C on solid M9 minimal medium supplemented with 10% potato extract (v/v). GusA activity was measured 24 h after inoculation using \( p \)-nitrophenyl \( \beta \)-D-glucuronide as substrate (Novel et al., 1974). The degradation product, \( p \)-nitrophenol (pNP), was detected at 405 nm, and specific GusA activity was expressed as nmol pNP liberated min\(^{-1}\) (OD\(_{405}\) unit\(^{-1}\)).

**Motility assay.** Motility was evaluated on LB soft-agar plates (0.3% w/v) agar). A sample (1 ml) of overnight culture of each strain was used to make a dilution in M9 to an OD\(_{600}\) of 2.0. Cultures were stabbed into the centre of soft-agar plates using a sterile inoculation needle. Plates were incubated at 30 °C for 24 h.

**Western blotting.** Cultures of P. w for Western blotting were grown at 30 °C in LB medium to an OD\(_{600}\) of 1.0 before induction with 0.1M IPTG. After 4 h induction, the cells were harvested by centrifugation at 4 °C and extracellular proteins were precipitated overnight at 4 °C with 5% (v/v) trichloroacetic acid (final concentration). The precipitates were isolated by centrifugation at 10 000 g for 20 min at 4 °C and the pellets were washed twice with ice-cold acetone, air-dried, and then resolubilized in 0.1M sodium phosphate buffer, pH 7.0. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad). Equal amounts of total protein (10 \( \mu \)g) were loaded on 12.5% SDS-polyacrylamide (w/v) gels and transferred to NitroBind nitrocellulose membranes (HybondTM -ECLTM, Amersham Biosciences). For Western blotting, the membranes were probed with a 1:2500 dilution of monoclonal antibodies raised against Nip\(_{P.w}\) in rabbits (LabAs) followed by treatment with goat anti-rabbit antibodies conjugated with alkaline phosphatase (Promega Biotech). The blots were developed using bromochloroindolyl phosphate/nitroblue tetrazolium.

**RESULTS**

**Generation of P. wasabiae flagellar mutants**

To identify gene(s) affecting nip\(_{P.w}\) expression, an rpoS mutant, SCC8003, which produces elevated levels of Nip\(_{P.w}\) (Mattinen et al., 2004), was subjected to transposon mutagenesis. For these studies, a total of 500 mutant colonies were grown individually and inoculated into tobacco leaf tissue. Only one mutant was associated with an absence of tissue necrosis on tobacco leaves (Fig. 1), and sequencing revealed a TnKm insertion in the intergenic
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli BL21(DE3)</td>
<td>$hsdS$ gal ($lacI857 ind1 Sam7 nin5 lacUV5-T7$ gene 1)</td>
<td>Studier &amp; Moffatt (1986)</td>
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<td>DH5-</td>
<td>$supE4, A LacU169, (lacZAM15)$, $hasdR17, recA1, endA1, gyrA96, thi-1, relA1$</td>
<td>BRL (San Diego, CA)</td>
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<td>P. wasabiae SCC3193</td>
<td>Wild-type strain</td>
<td>Pirhonen et al. (1988)</td>
</tr>
<tr>
<td>SCC8002</td>
<td>$rpoS:: Km$</td>
<td>Andersson et al. (1999)</td>
</tr>
<tr>
<td>SCC8003</td>
<td>$rpoS:: Cm$</td>
<td>Andersson et al. (1999)</td>
</tr>
<tr>
<td>SCC3300</td>
<td>$flgKL:: Km; rpoS:: Cm$</td>
<td>The present study</td>
</tr>
<tr>
<td>SCC3301</td>
<td>$flgKL:: Km$</td>
<td>The present study</td>
</tr>
<tr>
<td>SCC6028</td>
<td>$flhD:: Cm$</td>
<td>Andresen et al. (2010)</td>
</tr>
<tr>
<td>SCC6032</td>
<td>$flC:: Cm$</td>
<td>The present study</td>
</tr>
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<td>SCC6033</td>
<td>$flA:: Cm$</td>
<td>The present study</td>
</tr>
<tr>
<td>SCC6035</td>
<td>$outC:: Cm$</td>
<td>The present study</td>
</tr>
<tr>
<td>SCC6605</td>
<td>$resB:: Cm$</td>
<td>Andresen et al. (2007)</td>
</tr>
<tr>
<td>SCC6606</td>
<td>$resB:: Cm; flgKL:: Km$</td>
<td>The present study</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pMW119</td>
<td>Low-copy cloning vector (Amp$^R$)</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>pMW119gusA</td>
<td>Vector pMW119 containing the gus$^A$ gene in the HindIII site in the opposite direction from lacZ</td>
<td>Marits et al. (2002)</td>
</tr>
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<td>pKT3.1</td>
<td>Vector pMW119:: gus$^A$ containing the promoter region of nipP.w amplified from wt SCC3193 genomic DNA using primers NipP1 and NipP2 into the Smal digested plasmid</td>
<td>The present study</td>
</tr>
<tr>
<td>pGP704L</td>
<td>Delivery plasmid for homologous recombination (Amp$^R$)</td>
<td>Pavel et al. (1994)</td>
</tr>
<tr>
<td>PK3</td>
<td>Derivative of pANTSY containing a FLP recognition target (FRT)-flanked Cm$^R$ (cmt) gene from pSC140</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pNipP.w</td>
<td>Vector pBlueScript SK containing nipP.w in the Clal site</td>
<td>Mattinen et al. (2004)</td>
</tr>
<tr>
<td>pBRR1-lactac</td>
<td>Cloning vector containing lacZ gene and tac promoter (Gm$^R$)</td>
<td>R. Hörak (unpublished)</td>
</tr>
<tr>
<td>pKL-Gm</td>
<td>Vector pBRR1-lactac containing the flgKL amplified from wt SCC3193 genomic DNA using primers SalFlgK and flgKKpn gene between SalI and KpnI sites under the control of the tac promoter (Gm$^R$)</td>
<td>The present study</td>
</tr>
<tr>
<td>pKL</td>
<td>Plasmid pKL-Gm containing the Cm$^R$ gene amplified from the plasmid pKD3 DNA using primers CmP1 and CmP2 between BglII and Ncol sites (Cm$^R$)</td>
<td>The present study</td>
</tr>
<tr>
<td>pLK</td>
<td>Plasmid pKL-Gm containing the Cm$^R$ gene amplified from the plasmid pKD3 using primers CmP1 and CmP2 between BglII and Ncol sites (Cm$^R$)</td>
<td>The present study</td>
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</table>

region upstream of two flagellar genes, flgK and flgL. To characterize the effect of this flgKL mutation on nipP.w expression, a transcriptional fusion unit of nipP.w and gus$^A$ (pKT3.1) (nipP.w:: gus$^A$) was introduced into this rpoS/flgKL double mutant. Lower β-glucuronidase activity was produced by this double mutant than by the rpoS single mutant (Fig. 2). Furthermore, when the flgKL mutant was transduced into a wild-type background using T4GT, the activity of the nipP.w:: gus$^A$ transcriptional fusion was lower in the flgKL mutant than in the wild-type strain. The phenotypic responses elicited by expression of flgKL from a low-copy plasmid (pLK) restored nipP.w:: gus$^A$ (Fig. 2) expression and motility in an flgKL negative strain (Fig. 3).

To test whether mutations in other flagellar genes affect nipP.w expression, insertion mutants were generated in genes encoding the putative filament protein, flagellin (flIC) and in sigma factor σ28 (flhD). All of these mutants were non-motile on 0.3 % agar plates (Fig. 3).

**NipP.w:: gusA expression in flagellar mutants**

To characterize the influence of other flagella genes, the nipP.w:: gusA fusion was introduced into the flgKL, flIC, flIA and flhD mutants. As in the flgKL mutant, expression of the nipP.w:: gusA fusion was five to sixfold lower in the flhD mutant than in the wild-type strain (Fig. 4). In the flIA and
fliC mutants, expression of nipPw::gusA was comparable to the level of expression in the wild-type strain (Fig. 4). These results suggest that transcription of nipPw is not under the control of sigma factor σ28 in P. wasabiae, and that functional fliA and fliC genes important for motility are not involved in regulating nipPw transcription.

Involvement of the Rcs system in the regulation of nipPw

To test the hypothesis that an flgKL mutation affects nipPw expression via the Rcs system, expression of nipPw::gusA was measured in a wild-type strain, as well as in single mutants (e.g. rcsB, rcsD, rcsC and rcsF) and the double mutant (rcsBflgKL). While activation of rcsB had no effect on nipPw::gusA expression, nipPw::gusA expression was twice as great in the rcsB, rcsD and rcsC mutants as in the wild-type strain (Fig. 5). Furthermore, compared to rcsB mutants, the double mutant did not restore expression of the nipPw::gusA fusion. These results suggest that the negative effect of flgKL mutation on nipPw expression is only partly mediated through the Rcs system.

Secretion of NipPw protein in the wild-type strain versus different mutants

To test the hypothesis that mutations in flagellar genes affect NipPw secretion, the export of NipPw expressed

Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence (5'→3')*</th>
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<tr>
<td>FliCP1</td>
<td>TATCAACAGTCCAAAGACGATGCTGCGGGGCAGGCTATTGCAATCAACGGGCTGAGCTGCTTC</td>
</tr>
<tr>
<td>FliCP2</td>
<td>CGCCGTGTAAGTTGTTAGTGAAAGTACGCGGTCAATTTCATCTAAACCATATGAATATCCTCCTTAG</td>
</tr>
<tr>
<td>FliAP1</td>
<td>GCGCTATGTTCCACATGTGGCCATGAAGCCTTGGCTTTACAGGTTGTGTAGGCTGGAGCTGCTTC</td>
</tr>
<tr>
<td>FliAP2</td>
<td>ACGCGATAAGTGTGTAACGCCGTACCCTTGCAAAAGATACGCGGTCAATTTCATCTAAACCATATGAATATCCTCCTTAG</td>
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<tr>
<td>OutCP1</td>
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<tr>
<td>OutCP2</td>
<td>CGTAGCGGGCTTTTCTTTCGCTGGAGCGGCTAACAGACACACCGCAAAGATACGCGGTCAATTTCATCTAAACCATATGAATATCCTCCTTAG</td>
</tr>
<tr>
<td>NipP1</td>
<td>GCCATATCTACTGGAGTAAAGATACGCGGTCAATTTCATCTAAACCATATGAATATCCTCCTTAG</td>
</tr>
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<td>NipP2</td>
<td>GAAGATCTACTGCTGAGAAGTAAAGATACGCGGTCAATTTCATCTAAACCATATGAATATCCTCCTTAG</td>
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<td>SalFlgK</td>
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<td>flgLKpn</td>
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<td>SalFlgL</td>
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<td>flgKKpn</td>
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<tr>
<td>CmP2</td>
<td>CGAAAATGAGACGTTGAGATTTGCTG</td>
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*Sites designed for restriction enzymes are underlined.

Fig. 1. Induction of cell death in tobacco tissue by P.w wt, rpoS mutant, flgKL mutant and buffer control. The wt strain and the mutants were grown for three days at 15 °C on solid minimal induction medium (Huynh et al., 1989) supplemented with 10 mM manitol and 0.5% sucrose. Nicotiana tabacum cv. Samsun plants used in the HR assays were grown in greenhouses at approximately 22 °C. Leaves were photographed two days after infiltration.

Fig. 2. Expression of the nipPw::gusA fusion in wild-type and mutant strains. GUS activity associated with rpoS, flgKL-rpoS, flgKL and flgKL (pLK) mutants compared to wild-type. Strains were grown on minimal agar plates supplemented with 0.4% glycerol and 10% potato extract at 28 °C. Levels of GUS activity were measured 24 h after inoculation. Bars represent the average from three independent experiments, with standard deviations shown.
ectopically in flmA, flmC, flhD and flgKL flagellar mutants was examined. For these analyses, nipPw expression was placed under the control of a p lac promoter (pNipPw) in order to be independent of mechanisms affecting nipPw transcription, thus enabling the effects on NipPw secretion to be examined directly.

To determine the involvement of the flagellar secretion apparatus in NipPw secretion, the secretion of NipPw was examined in the flmA, flmC, flhD and flgKL mutants. Using immunoblotting, culture supernatants from the flmA, flmC, flhD and flgKL mutants were analysed. In all the samples studied, the amount of NipPw in the supernatant was comparable to that from the wild-type strain (Fig. 6). Taken together, these results suggest that NipPw is unlikely to be secreted through the flagellar type III secretion system in P. wasabiae.

In parallel, NipPw protein secretion was assayed in the outC mutant. As illustrated in Fig. 6, immunoblotting revealed almost undetectable levels of NipPw in the supernatant of the outC mutant. In combination, these results suggest that
a functional type II (Out-system) secretion system is required for \textit{Nip}_{\textit{P.w}} secretion.

**DISCUSSION**

When an \textit{rpoS} strain overexpressing \textit{Nip}_{\textit{P.w}} was subjected to mutagenesis, a mutant exhibiting reduced necrosis in tobacco tissue was identified. The relevant mutation was localized to the intergenic region upstream of two flagellar genes, \textit{flgK} and \textit{flgL}, suggesting that flagella are important for the production of \textit{Nip}_{\textit{P.w}}. To characterize the mechanism involved in the phenotype of the mutant further, several motility mutants were analysed.

The transposon insert caused a clear decrease in \textit{nip}_{\textit{P.w}} transcription in \textit{flgKL} (Fig. 2), but had no effect on the expression of PCWDE genes (data not shown). Without assembly of the FlgKL proteins, the HBB complex is most likely not functional and filament subunits are unable to polymerize. However, rather than a direct effect on \textit{nip}_{\textit{P.w}} transcription, it is hypothesized that inefficient assembly of the flagellar HBB complex or mislocation of the FlIC monomers due to the defective HBB complex leads to activation of the Rcs phosphorelay system and decreased \textit{nip}_{\textit{P.w}} transcription. The Rcs phosphorelay system has previously been identified as a repressor of genes responsible for the PCWDE synthesis (Andresen et al., 2007, 2010). The higher expression of the \textit{nip}_{\textit{P.w}}::\textit{gusA} fusion in the \textit{rcsBflgKL} double mutant than in the \textit{flgKL} mutant (Fig. 5) also suggests that a mutation in \textit{flgKL} affects \textit{nip}_{\textit{P.w}} transcription by modulating the activity of the Rcs phosphorelay system. The Rcs phosphorelay responds to a wide range of conditions, including structural perturbations in the peptidoglycan layer or cell membrane (Clarke, 2010). Our findings are also consistent with the observation that artificial membrane stress induced by deletion of the cytoplasmic membrane-anchored DjlA and outer membrane OmpG protein activates the Rcs phosphorelay system in different bacteria (Chen et al., 2001; Shiba et al., 2006). In addition, it has been demonstrated that the negative effects of the Rcs phosphorelay system on PCWDE in \textit{P. wasabiae} are achieved through \textit{rsmB} expression directly by binding the \textit{rsmB} promoter, or indirectly by reducing \textit{flhDC} transcription and increasing \textit{RsmA} levels in the cell (Andresen et al., 2010). These data are also consistent with the work of Pemberton et al. (2005), who observed a decrease in \textit{nip} expression in the absence of homoserine lactone production, which resulted in increased levels of \textit{RsmA}. On the other hand, increased production of \textit{Nip} in the \textit{rpoS} mutant could be caused by reduced levels of \textit{RsmA} (Mattinen et al., 2004). Therefore, it remains unclear whether \textit{RsmA} affects the stability of \textit{nip}_{\textit{P.w}} transcripts directly, or indirectly. The results of the present study, where expression of the \textit{nip}_{\textit{P.w}}::\textit{gusA} fusion in the \textit{rcsBflgKL} double negative mutant was lower than in the wild-type strain, suggest that mutation in flagellar genes is likely to trigger an additional Rcs phosphorelay-independent pathway to repress \textit{nip}_{\textit{P.w}} transcription. It is also possible that defects in HBB assembly activate an unknown regulatory system, which in turn activates the Rsm system. A possible mechanism leading to this phenotype could be feedback regulation of \textit{flhDC} due to defective flagellar assembly. However, there was no difference in \textit{flhDC} expression between the \textit{flgKL} mutant and the wild-type strain, suggesting that feedback regulation is not a likely explanation.

Recently, Cui et al. (2008) described a complex interplay between motility and bacterial virulence, where the master regulator of flagellar genes, \textit{FlhDC}, concomitantly controls flagella and PCWDE synthesis. Correspondingly, the results of the present study also demonstrate that \textit{FlhDC} is necessary for \textit{nip}_{\textit{P.w}} expression (Fig. 4). Furthermore, the present results also demonstrate that mutation in a gene involved in the assembly of HBB cause reduction in motility as well as decreases \textit{nip}_{\textit{P.w}} expression.

Mutations in \textit{fliA} (genes involved in the regulation of class III flagellar genes) and in \textit{flic} (a class III structural flagellar gene), resulted in non-motile cells (Fig. 3) in which \textit{nip}_{\textit{P.w}} expression was unchanged (Fig. 4). These results suggest that the \textit{fliA} and \textit{flic} mutants still produce a normal HBB, yet flagellar proteins do not accumulate inside the cell or in the periplasm. In addition, the cell membrane remains intact, thereby not activating the Rcs phosphorelay system and inhibiting \textit{nip}_{\textit{P.w}} expression.

It has previously been demonstrated that the bacterial flagellar export apparatus also functions as a secretion system for non-flagellar proteins (Young et al., 1999; Ghelardi et al., 2002). This ability of the flagellar export machinery to maintain two types of function supports the hypothesis that this system also contributes to \textit{Nip}_{\textit{P.w}} secretion. However, the detection of similar levels of \textit{Nip}_{\textit{P.w}} in immunoblots of supernatant samples from all flagellar mutants harbouring the \textit{nip}_{\textit{P.w}} gene under the control of the lac-promoter indicates that compromise of the flagellar export machinery (especially in \textit{flgKL} mutant) does not abolish \textit{Nip}_{\textit{P.w}} export. \textit{Nip}_{\textit{P.w}} contains a Sec-dependent
signal sequence, which allows it to be secreted through a type II secretion system. Coulthurst et al. (2008) also observed that Nip was secreted in an Out-dependent manner in Pectobacterium atroseptica. Taken together, the results of the present study and those of previous studies indicate that Nip_Pw, transported out of the cell via a type II secretion system (as are many other known virulence factors, e.g. cellulases and pectinases) in P. wasabiae (Reeves et al., 1994; Corbett et al., 2005). In summary, the present study provides evidence suggesting that the reduced virulence of flagella mutants may not be solely due to reduced motility, but also to reduced production of one or more virulence factors. In addition, although the mechanistic details of the effect of the flgKL mutation on nipPw, gene transcription were not completely identified, at least part of this pathway was found to involve the Rcs phosphorelay system.

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